HIV treatment with highly active anti-retroviral therapy cannot eliminate latent reservoirs of HIV. In a recent paper, Root and Hamer describe a novel targeting mechanism for killing HIV-infected cells. They show that 5-helix (a gp41-binding protein) fused to a Pseudomonas exotoxin fragment can selectively kill cells displaying envelope proteins on their surface from a broad range of HIV strains.

HIV/AIDS has been transformed from a fatal infection into a chronic infection for those who can afford and tolerate the current cocktail of reverse transcriptase and protease inhibitors. Although this treatment often reduces viral loads to undetectable levels, HIV lurks in poorly understood latent reservoirs, waiting to re-establish fulminant infection when the drugs are discontinued or resistance develops [1]. Consequently, there is great demand for an agent that will specifically kill HIV-infected cells. Root and Hamer [2] have delivered a potent toxin (Pseudomonas exotoxin fragment, PE) to HIV-infected cells that bear a highly conserved cell-surface marker of HIV infection (gp41). This approach has the potential to depopulate or even eliminate reservoirs of HIV infection.

HIV entry
HIV infection starts with the fusion of the viral and cell membranes, delivering the HIV genome into the host cell (Fig. 1). Fusion is mediated by the viral envelope glycoprotein (Env), which is composed of two non-covalently associated subunits – the transmembrane-anchored gp41 and surface-associated gp120. gp120 interacts with cellular receptors and gp41 mediates membrane fusion. gp41 contains two helical sequences at its N- and C-terminal ends (N- and C-peptide regions). Peptides corresponding to these helical regions (N- and C-peptides) inhibit HIV entry by preventing the N- and C-peptides from forming a trimer-of-hairpins.

Env first interacts with its target cell via CD4 and chemokine co-receptor (CCR5 or CXCR4) binding to gp120. This binding induces a series of large structural changes in gp120 and gp41 leading to the formation of a trimer-of-hairpins structure via a transient pre-hairpin intermediate (Fig. 1) [3]. Trimer-of-hairpins formation is thought to be the driving force for membrane fusion. According to this model, any molecule that binds to the transiently exposed pre-hairpin intermediate could prevent hairpin formation and therefore inhibit membrane fusion.

5-helix – a designed entry inhibitor
5-helix is composed of three N- and two C-peptides connected by slack Gly/Ser linkers (Fig. 2) [4]. It binds to the C-peptide region of gp41 very tightly [50% inhibition of entry (IC_50) at low nM concentration of inhibitor], reconstituting the stable 6-helix bundle. 5-helix is also a potent inhibitor of HIV entry, presumably by binding to the accessible C-peptide region in the pre-hairpin intermediate and preventing trimer-of-hairpins formation. Perhaps most importantly, the 5-helix binding site is highly conserved throughout a diverse group of laboratory and clinical HIV isolates.

Accessible targets in native gp41
In this study [2], Root and Hamer performed preliminary experiments to determine if the C-peptide region of gp41 (the target of 5-helix) is sufficiently exposed in the native state to be recognized by 5-helix. They found that 5-helix can precipitate a small (~1%), but reproducible, fraction of native gp41, in agreement with the recent results of Chan and co-workers [5]. As Root and Hamer discuss, this result is open to many interpretations. The C-peptide region might be largely exposed, with low precipitation efficiency caused by rigorous washing conditions and a large inaccessible reservoir of intracellular gp41. Alternatively, this small accessible fraction might represent unfolded or misfolded gp41 with the C-peptide region exposed. The ability of 5-helix to bind to the unfolded C-peptide [4] supports this explanation. Regardless of the explanation, a significant fraction of native gp41 on the cell surface is available for binding to 5-helix.

Special delivery of toxins
The targeted delivery of highly toxic agents to specific sites of disease has a long and checkered history [6]. The main challenge of this approach is achieving adequate specificity to avoid systemic toxicity, a level of specificity not easily achieved. Typically, a non-specific, highly cytotoxic agent (e.g. radioisotope, toxin or drug) is fused to a specificity domain. Many problems leading to systemic toxicity can occur with this strategy, including inadequate specificity, proteolysis between the specificity and toxin domains, and accumulation of toxin in sites of drug clearance (i.e. kidneys, liver and bone marrow). Antibodies are typically used to localize the toxin but, in principle, any agent that specifically accumulates at the desired target site could be suitable.

The PE fragment used in this study is widely used in a variety of experimental agents, particularly against
The highly toxic nature of the PE toxin means that only a small number of toxin molecules need to be delivered to kill a target cell. The PE fragment used in this study lacks a cell-targeting domain, therefore 5-helix is required to direct the toxic cargo to its site of action.

5-helix–PE as a targeted toxin

5-helix is a good candidate for delivering toxic agents to HIV-infected cells. It has extraordinarily high affinity for its target, which has no human homologs. The protein is compact (25 kDa) and does not interfere with the delivery of an attached PE fragment. Most importantly, 5-helix recognizes HIV-infected cells by the Env protein displayed on their surface. The 5-helix–PE toxin can potentially inhibit HIV infection in two distinct ways – inhibition of fusion by binding to the pre-hairpin intermediate, and cytotoxic activity delivered to the surface of infected (Env⁺) cells. These two mechanisms are complementary, attacking viral entry and production, respectively. Importantly, the fusion of 5-helix to the 38 kDa PE domain does not reduce the ability of 5-helix to inhibit entry [2].

As an initial test of 5-helix targeting ability, Root and Hamer showed that the 5-helix–PE fusion selectively kills Env⁺ cells (IC₅₀ of 1.9 nM versus >200 nM in Env⁻ cells) expressing Env from a laboratory-adapted strain. In a qualitative assay, 5-helix–PE also kills cells bearing Env from diverse HIV strains, with similar potency for all strains tested. These data correlate well with the experiments showing that 5-helix–PE binds to the surface of cells expressing these Env variants.
The applicability of these results is unclear because of the use of Env-overexpressing cells, which might be more vulnerable to an Env-directed toxin than authentic HIV-infected cells. To begin to address this concern, the ability of 5-helix–PE to prevent virion production in a population of partially infected cells was tested. 5-helix–PE was also an effective inhibitor in this assay (IC_{50} 2 nM), demonstrating that 5-helix–PE is also cytotoxic to cells expressing the lower levels of Env associated with active infected cells.

**Barriers to clinical use of 5-helix–PE**

This work represents an important proof of concept of the ‘targeted toxin’ approach for seeking and destroying latent reservoirs of HIV infection. However, there are many potential barriers to be overcome before these agents are used in the clinic. An important concern is that the level of Env expression on the surface of a quiescent infected cell is not well characterized and is probably too low to support 5-helix–PE activity and specificity [1]. Stability of the toxin fusion to proteolysis and immune clearance are also potential problems. Specificity (the ratio of activity of 5-helix–PE against Env^+ and Env^- cells) is fairly low in this study (~100-fold) and will probably be inadequate for avoiding unacceptable bystander toxicity. The fate of 5-helix–PE in the body is also a concern because its accumulation at sites of metabolism might be toxic. As with all recombinant therapeutics, 5-helix–PE will be costly to produce, particularly if chronic administration is necessary. Future work will need to address these limitations before 5-helix–PE or similar agents can be useful for treating AIDS.

**Comparison with other toxins targeted against latently infected cells**

Previous studies [8,9] have used a single-chain Fv (3B3) composed of only the variable regions of the heavy and light chains of the gp120-reactive antibody (b12) [10] as a targeting domain for PE. Like 5-helix–PE, 3B3–PE exhibits potent Env^+ cell killing and low non-specific toxicity in Env^- cells. Both 5-helix–PE and 3B3–PE toxins are active against a broad range of HIV strains. A potential advantage of targeting native gp120 is the universal availability of this target on the cell surface compared to the small number of gp41s accessible to 5-helix. A potential disadvantage is that gp120 sheds from cell or viral surfaces and could act as a decoy for 3B3. The relative utility of these two targeted toxins will depend largely on how easily resistance mutations develop and on their levels of non-specific toxicity.

An effective alternative for targeting HIV-infected cells was described by Dowdy and co-workers [11]. They fused a non-specific translocating peptide (Tat) to an inactive form of caspase (an apoptosis-inducing factor), which must be cleaved by HIV protease to become active. Although this protein can enter any cell, only those cells infected with HIV (and producing HIV protease) activate caspase, leading to apoptosis. This approach suffers from a similar potential limitation as the Env-directed toxins because HIV protease levels in latently infected cells might not be high enough to activate caspase.

**Future directions**

To overcome issues of potentially low Env concentration on the surface of infected cells, Root and Hamer propose combining 5-helix–PE with an activator of latent HIV. This strategy would help to reveal infected cells by increasing the expression (and surface display) of HIV proteins, but it carries the risk of worsening the infection.

Other future improvements might include stabilization of 5-helix–PE for a longer half-life in circulation, decreasing the immunogenicity of the 5-helix–PE fusion (e.g. via glycosylation), and possible combination of 5-helix–PE with other anti-fusion inhibitors to increase gp41 accessibility (e.g. soluble CD4). Combination of the 5-helix–PE toxin with therapeutics targeting other conserved HIV targets could also provide a boost to activity and specificity.

**References**